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CHARACTERIZATION OF TWO FORMS OF GLUCOAMYLASE FROM ASPERGILLUS NIGER

by

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Aspergillus niger glucoamylases GI and GII (E.C. 3.2.1.3) were isolated from a commercial enzyme preparation by ammonium sulfate precipitation followed by DEAE-cellulose ion exchange chromatography. Both enzymes consist of a single glycosylated polypeptide chain. The molecular weights of GI and GII were determined by sedimentation equilibrium ultracentrifugation to 52,000 and 46,000, respectively, and by molecular sieving to 65,000 and 55,000. The amino acid compositions of GI and GII were very similar. Furthermore, the N-terminal amino acid sequence of the intact GI and GII as well as of their cyanogen fragments were identical, suggesting great homology in the primary structure of the two forms. In addition the digests of GI and GII produced respectively by Armillaria mellea protease, Staphylococcus aureus V8 protease, and submaxillary protease were analyzed by high pressure gel permeation chromatography. The elution profiles were also consistent with GI and GII having similar polypeptide chains. However, digestion with carboxypeptidase Y showed different C-terminal residues of the two forms.

1. INTRODUCTION

Glucoamylase (1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.3) catalyzes the release of D-glucose from the non-reducing end of starch, glycogen and gluco-oligosaccharides. Although the α-1,6-glucosidic linkages are cleaved less readily than the α-1,4-glucosidic linkages (17, 28, 38), the debranching capacity of glucoamylases is sufficient to make them important in the industrial production of glucose from starch, a

Abbreviations: GI and GII denote two forms of glucoamylase.

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process of increasing interest in connection with ethanol production by fermentation (24, 49).

Glucoamylases are produced by a wide variety of microorganisms and exist frequently in multiple forms (13, 29, 33, 38, 48), one of which, generally referred to as GI, has the characteristic capacity to digest raw starch; in fact GI is able to completely convert starch into glucose (51). The structures of the multiple forms of A. niger glucoamylase have been shown to be closely related. However, conflicting results concerning the major difference between GI and GII have been reported; thus LINEBACK et al. (25, 27) have suggested that the forms differ in their polypeptide chain, whereas PAZUR et al. (35) claimed a difference only in the carbohydrate moieties. Also a variety of molecular weights are reported in the literature (9, 25, 34, 35, 43, 52).

In the present study the structures were compared of the glucoamylases GI and GII of A. niger, which were both isolated from a commercial enzyme preparation. The size and chemical composition of the molecules were reinvestigated and the GI form was found to digest raw starch. Furthermore, investigations of the amino acid sequence indicate that GI and GII have highly homologous primary structures.

2. MATERIALS

AMG 200 L was a commercial Aspergillus niger glucoamylase preparation obtained as a gift from Novo Industries, Bagsværd, Denmark. DEAE-Cellulose, DE 52, was from Whatman Ltd., Springfield, U.K. Bio-Gel P-100 was a product of Bio-Rad Laboratories, Richmond, Calif.

Iodoacetic acid, maltose monohydrate, soluble starch, and the kit »System Glucose« for glucose dehydrogenase determination of glucose were from Merck, Darmstadt, F.R.G. Transferrin (human), ovalbumin (Grade V), and dithiothreitol were purchased from Sigma, St. Louis, Miss. 2-Vinylpyridine was from Aldrich-Europe, Beerse, Belgium, and redistilled prior to use. β-Galactosidase (E. coli) and phosphorylase a (rabbit muscle) were from Boehringer, Mannheim, F.R.G., bovine serum albumin from Armour Pharmaceutical Comp., Eastbourne, U.K., chymotrypsinogen and ribonuclease from Worthington Biochem. Corp., Freehold, N.J., Staphylococcus aureus V8 protease from Miles, Stoke Poges, U.K., submaxillary protease from Pierce, Rockford, Ill., carboxypeptidase Y was a commercial preparation from United Breweries, Copenhagen, Denmark, and Armillaria mellea protease was a gift from Dr. V. B. PEDERSEN, Inst. Biochem. Gen., Univ. Copenhagen, Denmark.

Waxy maize starch, corn starch, wheat starch, rice starch, potato starch, glycogen, pullulan and 6-α-D-glucosyl-maltotriose were gifts from Dr. B. S. ENEVOLDSEN, Dept. Brew. Chem., United Breweries, Copenhagen, Denmark.

3. METHODS

3.1. Isolation of A. niger GI and GII

The GI and GII forms of glucoamylase of A. niger were purified by a modification of previously described procedures (27, 33, 52). All operations were carried out at 4 °C. The crude enzyme solution (200 ml) was centrifuged (30 min at 22.000 g) and to the supernatant was added ammonium sulfate to 60% saturation. The resulting precipitate was isolated by centrifugation (2 hours at 22.000 g), redissolved in water, adjusted to pH 7.0 with 1 M-NaOH, and dialyzed first against water and then 0.05 M-

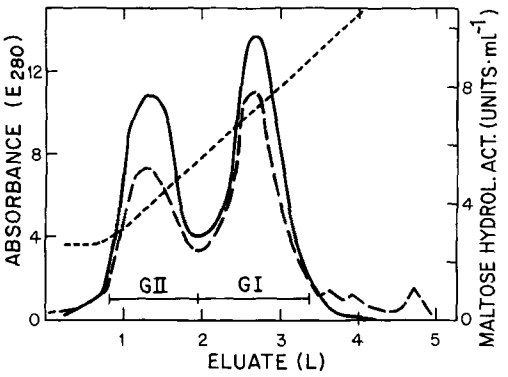

Figure 1. Separation of A. niger glucoamylases GI and GII, precipitated at 60% saturated ammonium sulfate, by ion exchange chromatography on DEAE-cellulose equilibrated with 0.05 M-sodium phosphate pH 7.0 and eluted by a linear gradient from 0 to 0.3 M-NaCl in the same buffer.

Absorbancy at 280 nm (— —), activity in units · ml^{-1} (——), and salt gradient (- - - -).

sodium phosphate pH 7.0. The sample was subsequently applied to a DEAE-cellulose column (5 × 30 cm), which had been equilibrated with 0.05 M-sodium phosphate pH 7.0. GI and GII were separated and eluted in high yields with a linear gradient from 0 to 0.3 M-NaCl in the same buffer (2 × 2.5 L) (Figure 1). The GI and GII pools were dialyzed against the starting buffer and rechromatographed under the same conditions. The pools of rechromatographed GI and GII were dialyzed against water and lyophilized. The enzyme preparations could be further purified by precipitation of GI and GII (5 $mg \cdot ml^{-1}$) at 65% or 85% saturated ammonium sulfate, respectively, followed by exhaustive dialysis against water and lyophilization. The recovery of GI varied from 75–100% and the recovery of GII at 75% saturated ammonium sulfate varied from 40–60%, whereas increasing the concentration corresponding to 85% saturation led to an additional recovery of 15–30% of GII. Alternatively, 1 g-portions of rechromatographed GI or GII could be purified by gel filtration on Bio-Gel P-100 (5 × 90 cm) in 0.05 M-sodium acetate pH 5.0. The salt was subsequently removed by dialysis against water and the enzyme recovered by lyophilization.

3.2. Analytical procedures

Electrophoresis in 10% polyacrylamide slab gels in the presence of dodecyl sulfate was performed by an earlier described modification (46) of the technique introduced by LAEMMLI (20). Molecular weights were estimated by calibration with β-galactosidase, phosphorylase a, transferrin, serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease. The protein samples were pretreated as described elsewhere (46), the thiol being omitted.

Polyacrylamide gel electrophoresis under non-denaturing conditions in 7% gels was performed a.m. DAVIS (4) at pH 8.3; the gels were stained for protein and carbohydrate (16).

Estimation of molecular size of GI and GII was carried out on a 1.5 × 90 cm Bio-Gel P-100 column in 0.05 M-sodium acetate pH 5.0 using serum albumin, ovalbumin, and chymotrypsinogen as molecular weight markers.

Sedimentation equilibrium measurements according to a modification of the meniscus depletion technique of YPHANTIS described by CHERVENKA (2) were performed at 29,500 $rev. \cdot min^{-1}$ and 20 °C in sodium chloride-sodium phosphate pH 7.0, I = 0.1 (30) in a Spinco Model E analytical ultracentrifuge. The partial specific volume of GI and GII was estimated from the amino acid and carbohydrate contents (3, 11) to 0.70 $ml \cdot g^{-1}$. Sedimentation velocity experiments were performed at 52,640 $rev. \cdot min^{-1}$ at 20 °C in the buffer described above at varying protein concentrations.

For automatic amino acid sequencing of GI and GII, the proteins were purified on Bio-Gel P-100 (see 3.1.), dissolved in 30% acetic acid (4–8 $mg \cdot ml^{-1}$) and 500 µl portions were analyzed in the Beckman Sequencer a.m. EDMAN and BEGG (8) under conditions described by JOHANSEN et al. (19). Phenylthiohydantoin amino acids were identified as described by SVENDSEN et al. (44). Prior to cyanogen bromide cleavage GI and GII were acetylated in 0.5 M-ammonium bicarbonate (17 $mg \cdot ml^{-1}$) by addition of 2–3 portions of distilled acetic anhydride (25 $\mu l \cdot ml^{-1}$) over a period of 1 hour. The pH was maintained about 8 by addition of aqueous ammonia, the modified proteins were recovered by lyophilization. Cyanogen bromide (10 mg) was added to 10 mg of modified GI and GII, dissolved in 1 ml of 70% formic acid, i.e. approx. 250 fold molar excess of reagent over methionine residues. After 20 hours at room temperature the reaction mixture was diluted with 10 volumes of water, lyophilized and subjected to sequencing.

Fragmentation of GI and GII using various proteases was carried out after carboxymethylation (18). The protein (10 $mg \cdot ml^{-1}$) was dissolved in 7 M-guanidinium chloride, 0.2 M-Tris HCl pH 8.4, nitrogen bubbled through for 30 min, and dithiothreitol in a 10 fold molar excess over half-cystines was added. After reduction for 2 hours at room temperature, a 1.2 fold molar excess of iodoacetate over sulfhydryls was added and the mixture left for 30 min before pH was adjusted to 3.5 by glacial acetic acid. The mixture was dialyzed against 0.1% acetic acid and subsequently lyophilized. Carboxymethyl-GI or -GII (2 $mg \cdot ml^{-1}$) were, respectively, dissolved in 0.05 M-ammonium bicarbonate and incubated with Armillaria mellea protease (20 $\mu g \cdot ml^{-1}$, 4 hours), submaxillary protease (20 $\mu g \cdot ml^{-1}$, 4 hours), or S. aureus V8 protease (25

$\mu g \cdot ml^{-1}$, 2 hours) at 37 °C (1, 23, 40). The reactions were stopped by boiling and the mixtures lyophilized. The digests were redissolved in 0.05 M-sodium phosphate pH 6.8 (4 $mg \cdot ml^{-1}$) and clarified by Millipore filtration. High pressure gel permeation chromatography was performed on 5–20 µl aliquots using a Waters high pressure liquid chromatograph equipped with a Waters protein column I-125. The flow rate was 0.5 $ml \cdot min^{-1}$ and the effluent was monitored at 206 or 220 nm.

For C-terminal analysis was used carboxypeptidase Y in 0.025 M-N-ethylmorpholine acetate pH 7.0, 0.2% in sodium dodecyl sulfate, at room temperature. The substrate concentration was 0.4 mM. Aliquots of 30 µl were removed at appropriate time intervals and the enzymic reaction was stopped by addition of 1 M-HCl, 30 µl. The mixture was evaporated to dryness and the amounts of free amino acids determined after application of the unhydrolyzed sample to the amino acid analyzer. Since asparagine, glutamine, and serine eluted essentially at the same position in the amino acid chromatogram the identification was performed after hydrolysis. For selected aliquots of incubation mixture the protein was precipitated in the cold by 90% ethanol and the contents of amino acids in the supernatant before and after hydrolysis were compared. Appropriate blanks containing substrate with carboxypeptidase Y omitted were run in parallel in order to establish a correction for the contribution to the amino acid contents due to small amounts of protein remaining in the supernatant.

Amino acid analysis was performed as described previously (45). For determination of the amino acid composition of GI and GII, samples were hydrolyzed 24, 48 or 72 hours. Half-cystines were determined after reduction with dithiothreitol followed by carboxymethylation (see above) or 2-vinylpyridinylation (10). Tryptophan was determined by the spectroscopic method of EDELHOCH (7).

Total carbohydrate was determined by the phenol-sulfuric acid procedure (6). For analysis of sugar in GI or GII, mannose, the predominant sugar residue (25, 36, 37), was chosen as standard. In order to remove free carbohydrate from the glycoprotein samples, 200 mg portions of rechromatographed GI or GII were dissolved in 10 ml of water, dialyzed against 1% glycine and subjected to preparative flat bed isoelectric focusing as described (53), in the pH range 2.5–4.5. The GI and GII bands were recovered and the Ampholines were removed as previously described (45). Alternatively, GI could be freed from non-covalently bound carbohydrate by addition of cold 20% trichloroacetic acid to a chilled solution of the protein (1 $mg \cdot ml^{-1}$) to a final concentration of 5%. The turbid solution was left overnight at 4 °C, the precipitate was collected by centrifugation and washed with 5% trichloroacetic acid. Carbohydrate and protein in the resuspended precipitate was determined by the phenol-sulfuric acid procedure and by amino acid analysis, respectively. GII did not precipitate upon addition of trichloroacetic acid.

Removal of the covalently bound carbohydrate by β-elimination under reducing conditions permitted estimation of the number of O-glycosylated serine and threonine residues in GI and GII (55). The glycoprotein (20 mg) was suspended in 0.1 M-NaOH, 0.3 M-sodium borohydride, for 24 hours at 45 °C. The pH was then adjusted to 5.0 by addition of glacial acetic acid, the samples were lyophilized, evaporated several times from methanol to remove methylborate, and finally subjected to hydrolysis and amino acid analysis as described above.

Concentrations of GI and GII were estimated from the UV-absorbance using $E^{1\%}_{280}$-values calculated by the aid of amino acid and carbohydrate analysis to be 18.7 and 17.9, respectively.

The enzymic activity was routinely measured with 0.015 M-maltose as substrate in 0.05 M-sodium acetate pH 4.3. One unit of glucoamylase was defined as the amount of enzyme which hydrolyzes 1 µmole of maltose per minute at 25 °C. After 30 minutes of incubation the enzymic reaction was stopped by addition of 1.5 volumes of 1.67 M-Tris pH 7.6. The glucose released was determined by the aid of the Merck »System Glucose« kit comprising glucose dehydrogenase, β-mutarotase, and NAD.

The enzymic hydrolysis of polysaccharides was investigated with different substrates. To suspensions of raw starch, 0.5% in 0.05 M-sodium acetate pH 4.3 (2 ml), was added 1 mg of GI or GII followed by incubation for 90 min at 25 °C under horizontal shaking. The reaction was stopped by addition of 1.67 M-Tris pH 7.6 (3

ml). Glycogen, soluble starch, or pullulan (0.5 % in 0.05 M-sodium acetate pH 4.3, 2 ml) were incubated for 30 min at 25 °C with 0.01–0.1 mg of GI or GII. The reaction was stopped and the glucose released was determined as described above. With polysaccharide substrates one unit of glucoamylase was defined as the amount of enzyme which releases 1 μmole of glucose per minute at 25 °C.

The hydrolysis of polysaccharide was also followed over a longer time period using gelatinized waxy maize starch as substrate (28). The starch (1 g) was dissolved in 0.5 M-NaOH (50 ml), the solution was heated to 80 °C, cooled to room temperature, adjusted to pH 4.3 by dropwise addition of 5 M-HCl and diluted with sodium acetate buffer pH 4.3 to 0.1 M, the resulting starch concentration being 9.1 mg · ml^{-1}. GI and GII (approx. 1 nmole) were used for digestion in 2.1 ml of this substrate at 37 °C. Aliquots (0.1 ml) were removed at suitable time intervals, the enzymic reaction stopped by addition of 1.67 M-Tris pH 7.6 (0.4 ml) and glucose and total carbohydrate determined.

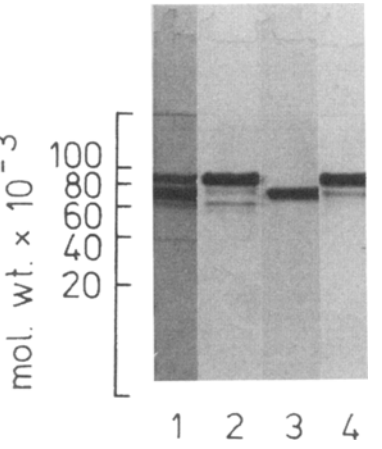

Figure 2. Gel electrophoresis in a 10% polyacrylamide slab gel in the presence of sodium dodecyl sulfate.

Lane 1: crude enzyme, 75 μg. Lane 2: rechromatographed GI, 75 μg. Lane 3: rechromatographed GII, 90 μg. Lane 4: rechromatographed GI purified further by preparative flat bed isoelectric focusing, 65 μg. The samples were dialyzed against 0.1 % sodium dodecyl sulfate, 0.01 M in Tris-HCl pH 6.5, mixed 1:1 with sample buffer and subjected to electrophoresis.

4. RESULTS

4.1. Isolation of A. niger glucoamylases GI and GII

The isolation procedure described in section 3.1 removed some colored material found in the concentrated culture filtrate. In 60% saturated ammonium sulfate about half of the maltose

Table I

Isolation of A. niger glucoamylases GI and GII

Step of purification	Activity[a] Units × 10^{-3}	Protein[b] mg × 10^{-3}	Yield (%)	Specific activity[c]
Crude enzyme (200 ml)	40.0	23.6	100	1.7
Redissolved $(NH_4)_2SO_4$-ppt.	21.2	12.2	53	1.7
DEAE-cellulose chromatography:				
GI-pool	9.9	6.6	25	1.5
GII-pool	8.8	4.9	22	1.8
DEAE-cellulose rechromatography:				
GI	8.9	5.4	22	1.6
GII	7.9	4.1	20	1.9

[a] One unit of enzyme activity catalyzes the hydrolysis of 1 μmole of maltose · min^{-1} at 25 °C and pH 4.3.
[b] Determined by amino acid analysis.
[c] Expressed as unit · mg^{-1} protein moiety.

hydrolyzing activity in the crude enzyme was obtained as a precipitate (Table I), which contained relatively higher amounts of GI in proportion to GII than in the crude enzyme. In the crude enzyme itself the ratio of GI to GII varied from 3.0 to 0.3 depending on the batch and the age of the batch. The glucoamylases were the major protein constituents in the crude enzyme preparation (Figure 2, lane 1) and the isolation of GI and GII was not accompanied by a significant increase in specific activity (Table I). A polypeptide component of apparent molecular weight 38,000 was removed by the ion exchange chromatography (Figure 2, compare lanes 1 and 2). Gel electrophoresis of large amounts of GI showed that the preparation contained an impurity of apparent molecular weight 61,000 (Figure 2, lane 2). In some preparations this band was very weak and disappeared after ammonium sulfate precipitation of rechromatographed GI or by preparative flat bed isoelectric focusing (Figure 2, lane 4). The GII form of glucoamylase appeared free from contaminating polypeptides after the ion exchange chromatography (Figure 2, lane 3). In concentrated samples of GI trace amounts of GII were detected, which may have been generated from GI during the sample pretreatment (Figure 2, lane 4).

4.2. Physico-chemical properties of A. niger glucoamylases GI and GII

The rechromatographed enzyme preparations appeared homogeneous by gel electrophoresis under non-denaturing conditions (Figure 3).

In sedimentation velocity ultracentrifugation experiments both GI and GII sedimented as single, symmetrical bands. The sedimentation coefficients, $s^{\circ}_{20,w}$, were 5.2 S and 5.1 S for GI and GII, respectively (Figure 5). Sedimentation equilibrium measurements with GI and GII at concentrations of about 2 mg·ml^{-1} did not indicate any inhomogeneities (Figure 4) and molecular weights of 52,000 and 46,000 were obtained for GI and GII, respectively.

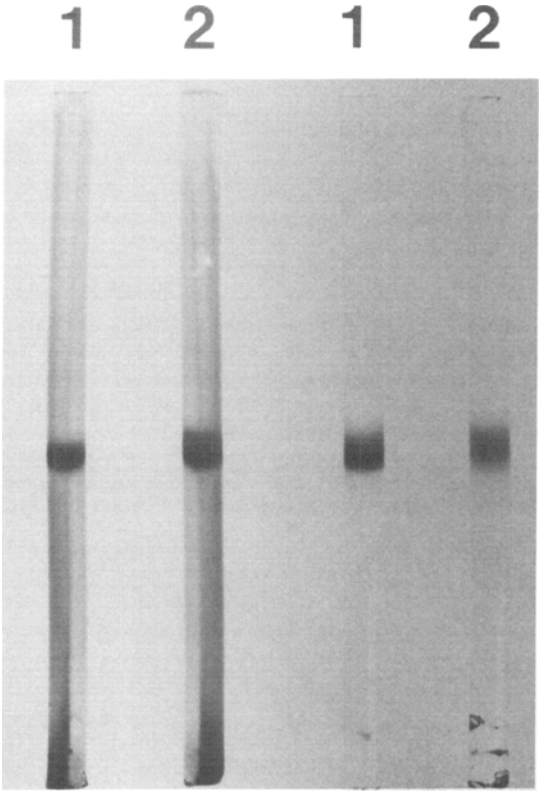

Figure 3. Electrophoresis in 7% acrylamide gels a.m. DAVIS (4).

15 µg of protein was applied. 1: GI, 2: GII. Protein staining to the left and carbohydrate staining to the right.

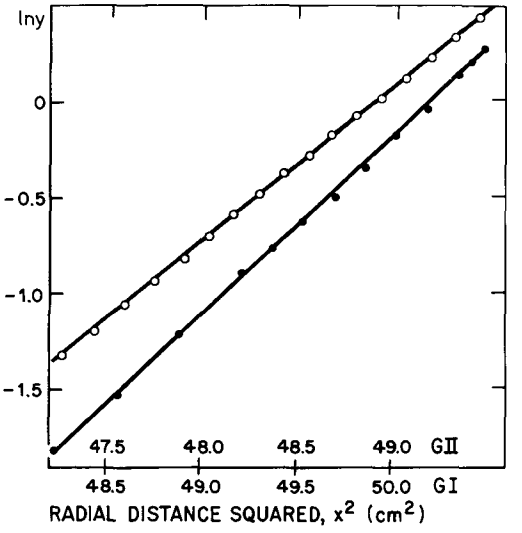

Figure 4. Sedimentation equilibrium of A. niger GI and GII.

Plot of the logarithm of the fringe displacement versus the radial distance squared. GI (●), 2 mg·ml^{-1}. GII (○), 2 mg·ml^{-1}.

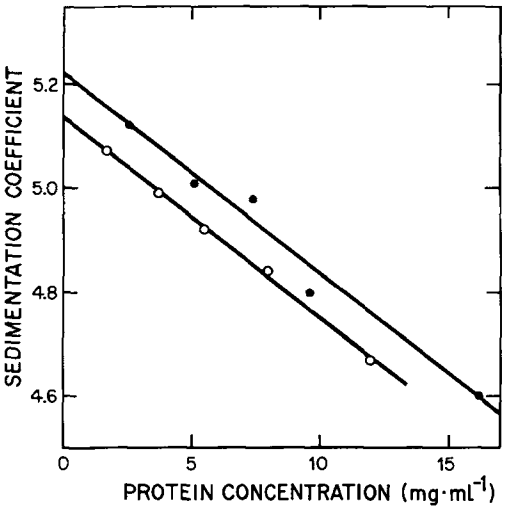

Figure 5. Sedimentation coefficient, $s_{20,w}$, of A. niger GI (●) and GII (○) as a function of the protein concentration.

The apparent molecular weight of enzymically active GI and GII was estimated by molecular sieving on Bio-Gel P-100 to 65,000 and 55,000, respectively, while apparent molecular weights of GI and GII were found to be 85,000 and 75,000, respectively, by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Figure 2). However, it is generally observed that glycopolypeptides under the conditions of the latter technique migrate at a slower rate than polypeptide chains of similar molecular weight (21, 22, 42).

In preparative flat bed isoelectric focusing GI gave a sharp major band of isoelectric point 4.0 and a minor one of 3.6. GII gave a single sharp band of isoelectric point 4.2.

4.3. Chemical properties of A. niger glucoamylases GI and GII

The amino acid composition of GI showed only minor differences from that of GII (Table II). Values reported for A. niger GI and GII by LINEBACK and AIRA (25) and PAZUR et al. (34, 35) are very close to those obtained in the present study, while greater variation was observed when comparing with the amino acid composition reported for A. niger glucoamylase by FREEDBERG et al. (9). In agreement with LINEBACK and AIRA (25) a half-cystine content of A. niger glucoamylase was determined to about 1.5 mole percent by dithiothreitol reduction followed by alkylation (Table II). However, half-cystines was determined after performic acid oxidation both in the present work and by PAZUR et al. (35) to about 1 mole per cent. An explanation for the variation in the contents obtained by reduction and alkylation with iodoacetate or 2-vinylpyridine as compared to performic acid oxidation is not known. Assuming two methionines and three histidines per molecule in both GI and GII the nearest integers for the amino acid residues per molecule could be calculated (Table II).

Table II

Amino acid composition of A. niger glucoamylases GI and GII

Amino acid	Moles of amino acid per 100 moles		Residues per molecule	
	GI	GII	GI	GII
Aspartic acid	11.1	11.2	49	44
Threonine	12.1	11.1	53	44
Serine	13.2	14.0	58	55
Glutamic acid	7.4	6.9	33	27
Proline	3.6	3.3	16	13
Glycine	7.5	7.7	33	30
Alanine	9.8	10.5	43	41
Valine	6.2	5.8	28	23
Methionine	0.43	0.45	2	2
Isoleucine	3.4	3.2	15	13
Leucine	6.8	7.1	30	28
Tyrosine	4.6	4.0	20	16
Phenylalanine	3.6	3.5	16	14
Histidine	0.68	0.77	3	3
Lysine	2.0	2.0	9	8
Arginine	2.7	2.9	12	11
Tryptophan	3.5	3.8	16	15
Cystine/2[a)]	1.3	1.5	6	6
Cystine/2[b)]	1.5	1.5	7	6
Total number			442	393

Averages of six amino acid determinations are reported. The serine and threonine contents were determined by extrapolation to zero time of hydrolysis, the valine and isoleucine contents are given for 72 h of hydrolysis. The number of half-cystine residues was determined for the reduced carboxymethylated (a) and 2-vinylpyridinylated (b) proteins. The tryptophan contents were measured by UV-spectroscopy.

The carbohydrate content of different GI preparations was estimated to 17–19% both after the pretreatment with trichloroacetic acid precipitation and after preparative isoelectric focusing. In the case of GII, which did not precipitate with trichloroacetic acid, the carbohydrate content after isoelectric focusing was determined to 22%. However, there is a possibility of carbohydrate inhomogeneity in GII, since 19–21% of carbohydrate was found in rechromatographed GII precipitated at 75% saturation of ammonium sulfate and 23–25% of carbohydrate was found after ammonium sulfate precipitation between 75 and 85% saturation.

The total contents of carbohydrate and amino acids of GI and GII corresponded to molecular weights of approximately 59,000 and 54,000, respectively, which is somewhat higher than the values obtained in sedimentation equilibrium analysis.

The enzyme preparations are glycosylated at serine and threonine residues as previously described for A. niger glucoamylase (5, 25, 34, 35). The approx. 30 O-glycosidically linked groups per molecule (Table III) corresponded to an average degree of polymerization of about two sugar residues. However, this number may be too large because vicinal glycosylated groups do not undergo complete β-elimination (31) and such sequences have recently been found in GI (unpublished observations).

4.4. Comparison of GI and GII by amino acid sequence analysis and peptide mapping

The primary structures of the two forms of glucoamylase were compared by amino acid sequence analysis from the N-terminus of the intact proteins and the 32 N-terminal residues of GI and GII were found to be identical (Figure 6). Some enzyme preparations were heterogeneous in the sense that about 50% of the polypeptide chains lacked the N-terminal tripeptide Ala-Thr-Leu, thus having an N-terminal aspartic acid residue.

Sequence analysis was also carried out on the mixture of fragments obtained by cyanogen bromide cleavage of acetylated GI and GII. The sequences corresponding to cleavage at the two methionines are shown in Figure 7. In formic acid methionyl-seryl peptide bonds are cleaved in low yield by cyanogen bromide, because the methionine is being converted to homoserine without the subsequent opening of the peptide bond (32, 41). The two sequences, therefore, were easily distinguished in the mixture, since the fragment with N-terminal serine only existed in 25–40% of the amount of the one starting with isoleucine. For the same reason the sequence of the peptide starting with serine could only be followed over seven steps, but again, the sequenced stretches of the fragments from GI and GII were identical.

Further evidence supporting an extensive similarity in the primary structure of GI and GII was obtained after digestion of the reduced, carboxymethylated proteins with proteases of

Table III

Reductive alkaline β-elimination of A. niger glucoamylases GI and GII

Amino acid	Residues per molecule					
	Before		After		Difference	
	GI	GII	GI	GII	GI	GII
Threonine	53	44	38	28	−15	−16
Serine	58	55	42	40	−16	−15
Glycine	33	30	34	32	+ 1	+ 2
Alanine	43	41	58	56	+15	+15
α-Aminobutyric acid	–	–	13	12	+13	+12

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Ala-Thr-Leu-Asp-Ser-Trp-Leu-Ser-Asp-Glu-Ala-Thr-Val-Ala-Arg-
 1                  5                  10                  15

Thr-Ala-Ile-Leu-Asn-Asn-Ile-Gly-Ala-Asp-Gly-Ala-Trp-Val-Ser-
                   20                  25                  30
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Figure 6. Amino acid sequence of GI and GII from the N-terminus of the intact proteins.

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Ile-Gly-Phe-Gly-Gln-Trp-Leu-Leu-Asp-Asn-Gly-Tyr-Thr-Ser-Thr-
 1                  5                  10                  15

Ala-Thr-Asp-Ile-Val-Trp-Pro-Leu-Val-Arg-Asn-Asp-Leu-Ser-Tyr-
                   20                  25                  30


Ser-Glu-Gln-Tyr-Asp-Lys-Ser-
 1                  5
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Figure 7. N-terminal amino acid sequences in mixtures of cyanogen bromide fragments from both acetylated GI and GII.

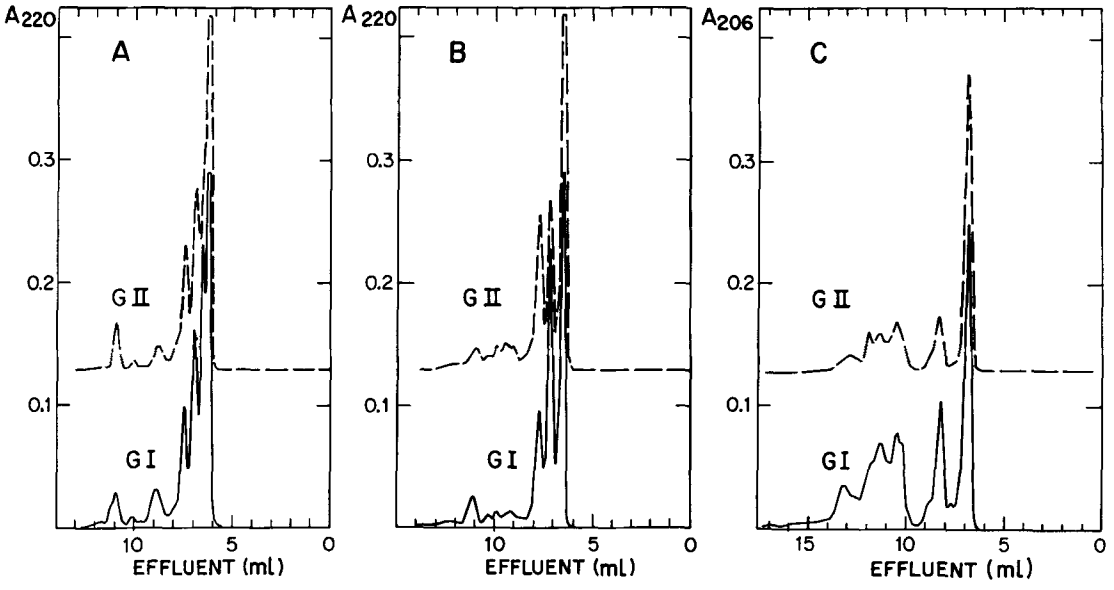

Figure 8. High pressure gel permeation chromatography of proteolytic digests of carboxymethylated GI and GII. Separation on a Waters protein column I-125 in 50 mM-sodium phosphate pH 6.8, monitored at 220 nm, of 40 μg GI (——) and GII (– – –) hydrolyzed by Armillaria mellea protease (A), submaxillary protease (B), S. aureus V8 protease (C, monitored at 206 nm). The curves from digests of GII are displaced by 0.125 absorbancy units.

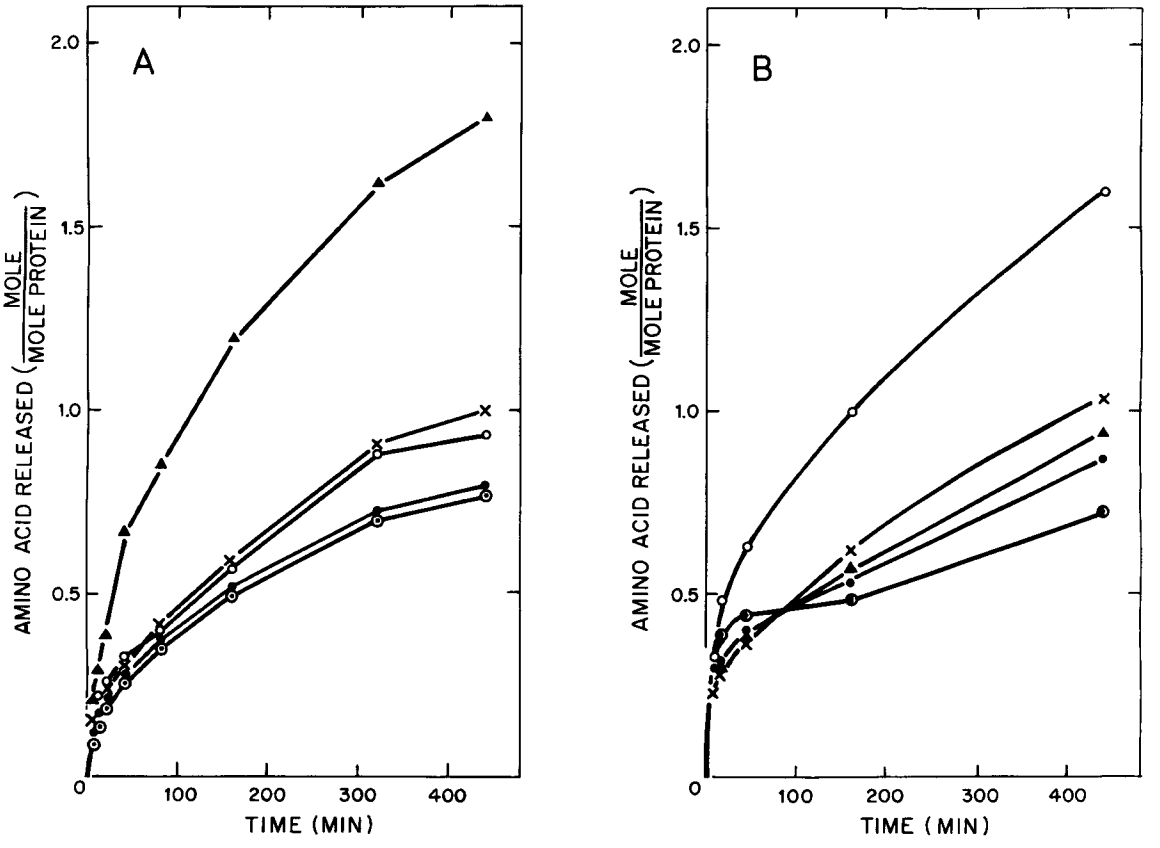

Figure 9. Release of free amino acids from GI (A) and GII (B) by carboxypeptidase Y.
Threonine (▲), alanine (×), serine (○), aspartic acid (●), valine (⊙), and leucine (◐). The conditions are described in section 3.2.

narrow specificity. Elution profiles from Armillaria mellea protease (Figure 8 A) and submaxillary protease digests (Figure 8 B) indicate that hydrolysis at the lysine and arginine residues, respectively, both resulted in larger amounts of long fragments as compared to cleavage by S. aureus V8 protease at the glutamic acid residues (Figure 8 C). Furthermore, typical patterns were obtained with the respective proteases and the GI and GII peptide mixtures seemed very similar. It is, however, difficult to predict to what extent small differences in the primary structure of homologous proteins are reflected in the corresponding elution profiles of fragment mixtures.

Analysis of the C-terminal end was carried out by the aid of carboxypeptidase Y. Clearly threonine was the residue first released from GI and serine from GII (Figure 9 A and B). GII may be heterogeneous at the C-terminal end since the shape of the curve describing the release of leucine could suggest that also a polypeptide with C-terminal leucine was found in the enzyme preparation (Figure 9 B). A longer C-terminal sequence cannot be interpreted with confidence for GI and GII, but the two forms are clearly different.

4.5. Enzymic properties of A. niger glucoamylases GI and GII

It has previously been demonstrated that the GI form of glucoamylases from Mucor rouxianus, A. cinnamomeus, A. oryzae (51) and A. awamori var. kawachi (15) could adsorb to raw starch and degrade this substrate, while the corresponding form, GII, attacked raw starch at

Table IV

Hydrolysis of raw starch by A. niger glucoamylases GI and GII

Substrate	Activity	
	GI	GII
Waxy maize starch	3.0	0.11
Corn starch	3.5	0.09
Wheat starch	3.5	0.25
Rice starch	3.0	0.31
Maltose	77	79

In order to facilitate comparison the activities are expressed in units per μmole of enzyme, where the molecular weights 59,000 and 54,000 were used for GI and GII, respectively.

Table V

Hydrolysis of soluble polysaccharides and oligosaccharides by A. niger glucoamylases GI and GII

Substrate	Activity	
	GI	GII
Soluble starch	220	150
Soluble potato starch	170	130
Glycogen	240	180
Pullulan	6.5	4.2
Maltose	77	79
6-α-D-glucosyl-maltotriose	64	67

In order to facilitate comparison the activities are given in units per μmole of enzyme, where the molecular weights 59,000 and 54,000 were used for GI and GII, respectively.

an extremely low rate. Similarly the GI form from A. niger has been shown in the present study to release glucose from raw starch, whereas GII had a very low activity on such substrates (Table IV). GII also hydrolyzed gelatinized waxy maize starch at a slightly slower rate as compared to GI (Figure 10). With both forms of glucoamylase, however, very close to complete conversion was attained with the final degree of hydrolysis being 96% and 92% for GI and GII, respectively. The difference in rate of hydrolysis was reflected also in the activity on α-glucans measured by the glucose released during 30 minutes of incubation. The activity of GII on the polymeric substrates was about 75% of the activity of GI, whereas the two forms were

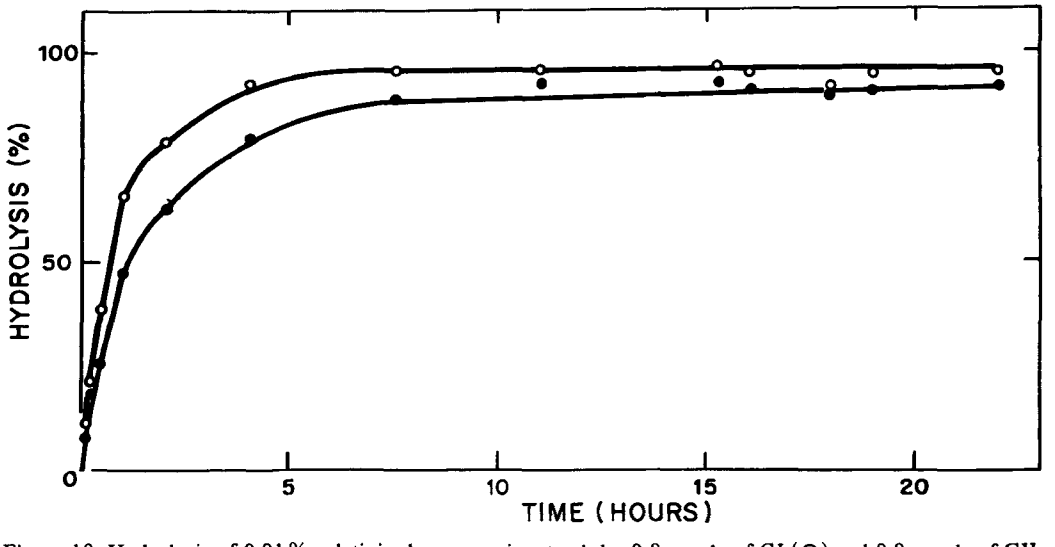

Figure 10. Hydrolysis of 0.91% gelatinized waxy maize starch by 0.8 nmole of GI (○) and 0.9 nmole of GII (●).

equally active in the hydrolysis of both α-1,4- and α-1,6-linkages in the low molecular weight substrates (Table V). The action of GI and GII on soluble substrates was in general in agreement with studies described in the literature (27, 43).

5. DISCUSSION

An increasing number of fungal glucoamylases has been proved to exist in multiple forms (38, 51). For each of the black molds A. niger (9, 27, 33, 52), A. awamori var. kawachi (13), A. saitoi (47), and A. phoenicis (26) two or more forms were easily separated by ion exchange chromatography. The multiple forms of glucoamylase from other molds than A. niger were previously shown to be distinguished in their function, the GI form having substantial activity toward raw starch in contrast to the GII form. In the present study the GI form of A. niger was similarly found to digest raw starch at a rate 10–30 times higher than GII.

Conflicting conclusions are reported in the literature on the structural relationship of GI and GII. Thus LINEBACK et al. (25, 27) found GI and GII to cross-react immunologically and to have the same total amount of carbohydrate, identical N-terminal amino acid residues, slightly different amino acid compositions, and molecular weights of 74,900 and 54,300, respectively. In contrast PAZUR et al. (35) found GII to contain twice the amount of carbohydrate as compared to GI, but again GI and GII cross-reacted immunologically, had identical N-terminal amino acid residues and also identical amino acid compositions, their molecular weights were 99,000 and 112,000, respectively. Actually, in the literature GI molecular weights from 63,000 to 110,000 and GII molecular weights from 53,000 to 112,000 are reported (9, 25, 34, 35, 43, 52). In all but one case (35) the form with the higher isoelectric point, GII, was found to be the smaller one. In the present study the molecular weights of GI and GII determined by sedimentation equilibrium centrifugation and molecular sieving were in the ranges 52,000–65,000 and 46,000–55,000, respectively. Thus the molecular weight of glucoamylase appears to be about 60,000 rather than 100,000 which is in agreement with the chemical composition of the enzyme. In contradistinction to FREEDBERG et al. (9) no sign of a subunit structure was found in the present study by sedimentation equilibrium or electrophoresis in dodecyl sulfate.

The N-terminal alanine previously found by LINEBACK et al. (27) and PAZUR et al. (35) was confirmed, whereas an N-terminal phenylalanine or a blocked N-terminal as reported by VENKATARAMU et al. (52) and FREEDBERG et al. (9), respectively, were incompatible with the present results. The structural studies of GI and GII have in the present work been extended to include comparison of sections of the primary structures. Both partial amino acid sequencing and peptide mapping indicated that the polypeptide chains of the two forms were very similar. Thus sequencing of analogous stretches in GI and GII, corresponding to about 13% of the polypeptide chain, gave identical results with both forms. Furthermore, only small differences were detected in the elution profiles from high pressure gel permeation chromatography of proteolytic digests of GI and GII. However, since GI and GII have different C-terminal residues and GI is the largest of the two molecules, it is likely that GI and GII contain highly homologous polypeptide chains, which in the case of GI extend C-terminally beyond the end of GII. In support of this suggestion it can be mentioned that in vitro stepwise degradation of GI glucoamylase from A. awamori var. kawachi (mol.wt. 90,000) using proteases and glycosidases, led to multiple forms with new C-terminal residues (mol.wts. 83,000 and 57,000), which had lost the ability to attack raw starch (13–15).

The glucoamylase from A. niger belongs together with the cellobiohydrolase C from Trichoderma viride (12) and the mycodextranase from Penicillum melinii (39, 50) to a class of unusual glycoproteins characterized by a high number of O-glycosylated amino acid residues, each carrying a short carbohydrate chain. The carbohydrate structure of glucoamylase GI from A. niger has recently been described in detail (5, 37). These and earlier chemical studies agree on an average chain length of the carbohydrate substituents corresponding to about two sugar residues, but variations exist in the number of carbohydrate chains reported per enzyme molecule (25, 35, 52). The results of the present study differ from LINEBACK and AIRA (25) in the degree

of glycosylation, but agree in GI and GII both having 60–65 sugar residues per molecule. In contrast, PAZUR et al. (35) report very different carbohydrate contents for GI and GII, i.e. 87 and 162 sugar residues per molecule, respectively. In the present study the carbohydrate moiety seemed very similar for the GI and GII forms, but a detailed structural investigation has not been performed. Hence, at present it cannot be decided whether the different enzymic behaviour of GI and GII is due primarily to the established difference in the polypeptide chain or it is associated also with a difference in the carbohydrate structure.

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